Biochemical and immunochemical analysis of the arrangement of connexin43 in rat heart gap junction membranes

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Summary

A 43×10^3 M_r protein (designated connexin 43 or Cx43) is a major constituent of heart gap junctions. The understanding of its arrangement in junctional membranes has been extended by means of site-directed antibodies raised against synthetic peptides of Cx43. These represent part of the first extracellular loop (EL-46), the cytoplasmic loop (CL-100), the second extracellular loop (EL-186) and carboxy-terminal sequences (CT-237 and CT-360). All of the antibodies raised reacted with their respective peptides and the Cx43 protein on Western blots. By immunoelectron microscopy two of the antibodies (CL-100 and CT-360) were shown to label the cytoplasmic surface of isolated gap junction membranes. Immunofluorescent labeling at locations of neonatal cardiac myocyte–myocyte apposition required an alkali/urea treatment when the EL-46 and EL-186 antibodies were used. Immunoblot analysis of endoproteinase Lys-C-digested gap junctions revealed that the Cx43 protein passed through the lipid bilayer four times. Alkaline phosphatase digestion of isolated junctions was used to show that the CT-360 antibody recognized many phosphorylated forms of Cx43. Our results unequivocally confirm models of the organization of Cx43 that were based on a more limited set of data and a priori considerations of the sequence.

Key words: antibodies, connexin43, gap junctions, myocytes.

Introduction

Gap junctions provide the means for the passage of small cytoplasmic molecules (e.g. inorganic ions, metabolites, messengers) from one cell to another. They consist of channels (connexons) that cross the membrane of one cell and pair with connexons from another cell, thus forming a pathway for direct cell–cell exchanges. Gap junctions are commonly thought to play roles in cell growth and differentiation (Warner *et al.* 1984; Revel, 1986; Gilula, 1987). In cardiac tissue, gap junctions are also believed to be involved in conductance of electric signals between cells, resulting in the synchronous beating of the heart (Griepp and Bernfield, 1978; De Haan *et al.* 1981; as reviewed by Forbes and Sperelakis, 1985).

A wealth of information has identified a 43×10^3 M_r protein (connexin43 or Cx43) as a major constituent of gap junctions in the heart (Manjunath *et al.* 1984*a*,*b*; Manjunath and Page, 1986; Beyer *et al.* 1987). Immunocytochemical labeling studies have provided direct evidence that Cx43 is found in gap junction membranes and that it is also widely distributed in other tissues and vertebrate species (Dupont *et al.* 1988; Beyer *et al.* 1989; Yancey *et al.* 1989). Evidence supporting a role for Cx43 in intercellular communication has recently been provided by Swenson *et al.* (1989), who showed elevated levels of gap junctional coupling in *Xenopus* oocyte pairs injected with Cx43-coding mRNA. The ability of several anti-connexin antibodies to block dye coupling (Hertzberg *et al.* 1985; Yancey *et al.* 1989) and electrical coupling (Hertzberg *et al.* 1985) after intracellular injection into cardiac myocytes has provided further support for the junctional role of Cx43. The rat Cx43 protein has been cloned (Beyer *et al.* 1987) and shown to have high sequence homology to the rat Cx32 (Paul, 1986) and Cx26 (Nicholson and Zhang, 1988; Zhang and Nicholson, 1989) gap junction proteins, both of which are found in the liver and other tissues.

The evidence adduced up to now suggests that the polypeptide chain of the Cx43 protein threads through the lipid bilayer four times with two loops exposed in the extracellular gap. These extracellular loops are thought to be responsible for the interactions between connexons in apposed cell membranes. The amino and carboxy termini are believed to be located in the cytoplasm (Manjunath *et al.* 1987; Beyer *et al.* 1987). An amino-terminal-specific, anti-Cx43 antibody, was recently used to confirm the cytoplasmic orientation of the N-terminal domain (Yancey *et al.* 1989). The same authors also provided experimental evidence for a cytoplasmic loop domain of approximately 4×10^3 M_r in size. This is consistent with models of the Cx32 protein, which is also believed to span the membrane four times and to have a cytoplasmic loop region and the carboxy terminus exposed to the cytoplasm (Zimmer *et al.* 1987; Hertzberg *et al.* 1988; Goodenough *et al.* 1988; Milks *et al.* 1988).

As an extension of earlier work on the topology and structure of Cx43 described above, five new site-directed antibodies were successfully raised to two highly conserved regions of the molecule predicted to represent the extracellular loops (Beyer *et al.* 1987) and three cytoplasmic domains (Beyer *et al.* 1987; Yancey *et al.* 1989). We have used proteolysis of the Cx43 protein in conjunction

with immunoblotting techniques to provide direct evidence that the polypeptide passes through the membrane four times, yielding two extracellular loops and an antibody-accessible cytoplasmic loop. Furthermore, our studies provide sharper constraints than available previously on what parts of the molecule could be in the membrane.

Materials and methods

Peptide synthesis

Peptides corresponding to the amino acid residues 46–76, 100–122, 186–206, 237–259 and 360–382 of Cx43 heart gap junction protein (Beyer *et al.* 1987) were synthesized on an Applied Biosystems Peptide synthesizer (Suzanna Horvath, Caltech). The purity of each peptide was determined by high performance liquid chromatography (HPLC). Four of the peptides (46–76, 100–122, 186–206 and 237–259) were synthesized with a cysteine added on at the carboxy terminus and one peptide (360–382) had a cysteine added on at the amino terminus. Approximately 12 mg of the reduced 237–259 and 360–382 peptides were conjugated through the carboxy and amino cysteines, respectively, to 10 mg of keyhole limpet hemocyanin (KLH) using succinimidyl-4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (Pierce Chemical Co., Rockford, IL) as a cross-linking reagent. Samples of the conjugated peptides were stored at −80 °C for future immunizations.

Production of site-directed polyclonal antibodies

Prior to the immunization of rabbits for antibody production, 10–20 ml of preimmune serum was collected from each rabbit and immediately stored at −80 °C for future use. Approximately 2 mg of free peptide (46–76, 100–122, 186–206) or 2.5 mg of KLH-conjugated peptide (237–259, 360–382) was dissolved in 2 ml of phosphate-buffered saline (PBS), pH 7.4, and reconstituted in a vial of adjuvant (Ribi, Hamilton, MT, Cat. no. R-730). The vial was vortexed extensively and 1 ml of the emulsion was administered to the rabbit by a series of intradermal, intramuscular and subcutaneous injections. The immunized rabbits were boosted at 3-week intervals with 1 ml of the same antigen using an identical injection protocol. Routinely, the serum from immunized rabbits was collected 7, 11 and 14 days after each boost and stored at −80 °C.

Antibody binding assay

A solid-phase radioimmune assay (RIA) was used to determine the ability of a rabbit immune serum to bind to immobilized synthetic peptides. Approximately 30–50 ng of synthetic peptides were immobilized in poly-L-lysine (average molecular weight 59 000) coated, flexible, 96-well assay plates (Falcon, Lincoln Park, NJ). The plates were quenched of non-specific binding sites by incubating the wells in PBS containing 1 % BSA for 1 h. The rabbit serum was serially diluted and 50 μl was added to each well for 1 h at room temperature. After extensive washing in PBS, antibody binding was detected by incubating the wells for 1 h in ^{125}I-labeled goat anti-rabbit IgG (1×10^6 to 2×10^6 disints μg^{-1}) antibody. After washing extensively in PBS, the radioimmune assay was completed by counting the individual wells in a gamma counter.

Antibody affinity purification

Site-directed antibodies were purified from rabbit sera according to the following procedure. Approximately 2–3 mg of synthetic peptide was dissolved in 0.7 ml of 0.5 M sodium carbonate buffer, pH 9.3, and incubated with an aldehyde-activated MAC 25 membrane filter (Memtec Corporation, Billerica, MA) overnight at room temperature. The filter was quenched of any remaining binding sites with 0.1 % sodium borohydride in PBS for 30 min and subsequently washed sequentially with PBS, 1 M NaCl, 0.1 M glycine, pH 2.3, and PBS. Approximately 1–3 ml of rabbit immune serum was passed through the filter several (10–15) times to allow for specific antibody binding. After washing the filter with 30–50 ml of PBS, bound antibody was eluted with 1–2 ml of 0.1 M glycine, pH 2.3. The absorbance at 280 nm was measured and the concentration of antibody was calculated using an extinction coefficient (mg^{-1} ml cm^{-1}) of 1.4. The pH of the solution containing the purified antibody was brought to neutrality by dialysis against PBS.

Conjugation of rhodamine to a purified site-directed antibody

Briefly, 1.5 ml (125 $\mu g\,ml^{-1}$) of affinity-purified CT-360 antibody in 0.1 M sodium carbonate (pH 9.0) containing 0.5 % BSA was combined with 50 μg of dimethyl sulfoxide (DMSO) dissolved tetramethylrhodamine isothiocyanate (Calbiochem Corporation, San Diego, CA) and incubated at 4 °C for 18 h. The conjugation reaction was stopped by incubating the mixture for an additional 2 h at 4 °C in the presence of 50 mM NH_4Cl. Finally, the unbound dye was separated from the conjugate on a Biogel P-4 column as described by Harlow and Lane (1988). The conjugate was stored in the dark at 4 °C for future immunofluorescent labeling studies.

Membrane preparations and proteolysis

Crude cardiac cell plasma membranes were prepared as described by Manjunath *et al.* (1985). Gap junction plaques were isolated from 300–350 g male Sprague-Dawley rat (Simonsen Laboratories, Gilroy, CA) hearts according to the method of Manjunath and Page (1986). The purity of the gap-junctional membrane preparations was assessed by SDS–PAGE in conjunction with Coomassie Blue staining. Protein concentrations were estimated by densitometry of Coomassie Blue-stained gels or by using BCA protein assay reagent (Pierce Chemical Co.).

Isolated cardiac gap-junctional plaques were digested with endoproteinase Lys-C (Boehringer-Mannheim Biochemicals, Indianapolis, IN) according to Yancey *et al.* (1989). Completely digested gap-junctional membranes were subjected to SDS–PAGE and Western blotting for analysis by purified antibodies.

Alkaline phosphatase digestion of isolated cardiac gap junctions

Isolated cardiac gap junctions were incubated with alkaline phosphatase from calf intestine (Boehringer-Mannheim Biochemicals) (4–7 units μg^{-1} gap junction protein) at 37 °C in 0.1 M Tris buffer, pH 8.0. After 2 h the junctions were centrifuged in an Eppendorf centrifuge for 15 min to remove the free phosphate. In order to ensure complete digestion, the gap junction pellet was resuspended in alkaline phosphatase (4–7 units μg^{-1} gap junction protein) and incubated at 37 °C for 14–16 h. The digested junctions were washed, solubilized and immediately applied to a 10 % SDS–polyacrylamide gel.

SDS–polyacrylamide gel electrophoresis and Western blotting

Gap junction membrane preparations were solubilized in an equal volume of denaturing buffer containing 4 % SDS, 10 % 2-mercaptoethanol, 0.01 % Bromophenol Blue and 60 mM Tris–HCl, pH 6.8. Generally, 5–10 μl of sample was applied to the wells of a 12 % SDS–polyacrylamide gel or a 10 % SDS–polyacrylamide gel containing 0.4 % bisacrylamide and electrophoresis was carried out at 15 mA using the buffer system of Laemmli (1970). Molecular weight standards obtained from Bio-Rad Laboratories (Cambridge, MA) were as follows: myosin, 200 000; phosphorylase B, 97 400; bovine serum albumin, 66 200; ovalbumin, 42 700; carbonic anhydrase, 31 000; soybean trypsin inhibitor, 21 500; and lysozyme, 14 400.

SDS-denatured proteins were transferred to nitrocellulose paper by a semi-dry transfer method as described by Yancey *et al.* (1989). Following transfer the nitrocellulose was quenched of any remaining protein binding sites by incubation in a Tween 20 immunoblot buffer (0.15 M NaCl, 1 mM EDTA, 1 mM NaN_3, 0.5 % Tween 20 and 20 mM Tris–acetate, pH 7.4). All subsequent antibody dilutions and washes were done in immunoblot buffer

containing 0.05% Tween 20. The quenched nitrocellulose blots were treated with 0.2–1.5 $\mu g\,ml^{-1}$ of purified site-directed antibodies for 1 h at 23°C. After extensive washing, antibody binding was detected using goat anti-rabbit IgG conjugated to alkaline phosphatase (Promega Biotec, Madison, WI). Finally, the blots were washed and developed according to established procedures using bromochloroindolyl phosphate/nitro blue tetrazolium substrate (Harlow and Lane, 1988).

Immunogold labeling of cardiac gap junction membranes

Approximately 0.5–1.0 μg of a gap junction membrane preparation was combined with 10–15 μg of a crude cardiac plasma membrane preparation in PBS and incubated overnight at room temperature in poly-L-lysine-coated 96-well plates. After rinsing out the unbound membranes the wells were quenched for 1 h in PBS containing 5% BSA. The immobilized membranes were treated with a 25- to 100-fold dilution of preimmune or immune serum for 1 h. Alternatively, the membranes were treated with 80–115 $\mu g\,ml^{-1}$ affinity-purified antibodies. After washing in PBS, the wells were treated for 1 h with a 10-fold dilution of goat anti-rabbit IgG antibody-conjugated to 5 nm gold particles (Janssen Life Sciences, Piscataway, NJ). After washing as before in PBS, the membranes were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, and prepared for electron microscopy as described by Yancey *et al.* (1989). Thin sections were viewed under a Philips 420 electron microscope.

Neonatal cardiac cell cultures

Primary cultures of neonatal rat heart myocytes were prepared according to a modification of Burt (1982) and Yancey *et al.* (1989). Hearts from one-day-old rats were dissected, minced and the cells were dissociated by a series of 15 min digests with 0.175% pancreatin (Gibco Laboratories, Grand Island, NY) at 37°C in 136 mM NaCl, 2.6 mM KCl, 0.3 mM NaH_2PO_4, 11.9 mM $NaHCO_3$, 11.1 mM dextrose and 0.25% Phenol Red, pH 7.2. Fully dissociated cells were plated in Hank's balanced salt solution supplemented with (GIBCO) 1×MEM vitamins, 1×MEM amino acids, 1×MEM non-essential amino acids, 2 mM L-glutamine, 0.66 mM glycine, 0.9 mM hypoxanthine, 9.8 mM $NaHCO_3$, 0.25% Phenol Red, 10% fetal calf serum and antibiotics. Cardiac myocytes were separated from more adhesive cells (fibroblasts) by plating the cells at 37°C for 60–90 min. The loosely attached myocytes were removed from the plate by gentle aspiration and 2×10^5 to 8×10^5 cells ml^{-1} were recultured in plastic dishes containing 12 mm glass coverslips. Cell cultures were maintained for 1–6 days at 37°C in an environment of 5% CO_2 and 95% air.

Immunofluorescent labeling studies

Myocytes grown on glass coverslips from 2- to 4-day-old cultures were rinsed in PBS and fixed in absolute ethanol for 10–20 min at room temperature. Alternatively, myocytes were fixed in 1% paraformaldehyde for 45–90 min at 4°C prior to washing in several changes of PBS for 30–45 min. In some cases, fixed cells were left at 4°C overnight in PBS and treated further with 10 mM Tris, pH 12.0, containing 8 M urea for 1–2 h at 30–37°C in an attempt to split the gap junction membranes. All samples were quenched of remaining non-specific binding sites by incubating in PBS containing 2% BSA for 45 min. Quenched coverslips were treated for 1 h with 2–20 $\mu g\,ml^{-1}$ affinity-purified site-directed antibodies diluted in quenching buffer. The cells were washed extensively in PBS and incubated for 1 h with a 500-fold dilution of a goat anti-rabbit IgG antibody conjugated to fluorescein (Boehringer-Mannheim). After washing as before, the coverslips were mounted in 90% glycerol containing 0.1% paraphenylenediamine and 100 mM Tris, pH 9.0.

In some cases, myocytes were labeled with culture fluid from an anti-myosin hybridoma cell line followed by a goat anti-mouse IgG antibody conjugated to rhodamine (Boehringer-Mannheim). These same cells were subsequently labeled with an anti-Cx43 antibody as described above. In other double-labeling experiments, permeabilized myocytes were indirectly treated with CL-100 antibody in conjunction with a goat anti-rabbit IgG fluorescein conjugate and labeled with CT-360 antibody directly conjugated to rhodamine. All cells were observed and photographed under a Zeiss phase-contrast microscope equipped for epifluorescence.

Results

Characterization of site-directed antibodies

The rat cardiac Cx43 gap junction protein consists of a single polypeptide chain of 382 amino acids (Beyer *et al.* 1987). Five peptide analogues to segments of this protein have been synthesized, purified and injected into rabbits for the production of site-directed antibodies. The peptide sequence and the assigned name for each antibody are shown in Table 1. The five peptides selected were predicted to be part of segments believed to be exposed outside the hydrophobic core of the Cx43 protein (Beyer *et al.* 1987; Yancey *et al.* 1989) (Fig. 1). Thus, the 46–76 and 186–206 peptides have been postulated to represent portions of the first and second extracellular loops, respectively, while the 100–122 peptide has been predicted to constitute part of the cytoplasmic loop. The 237–259 and 360–382 peptides, in turn, are believed to correspond to sequences located on the proteolytically sensitive carboxy-terminal portion of the molecule (Manjunath *et al.* 1985; Manjunath *et al.* 1987) (Fig. 1). The antibodies raised against these peptides were used to study the structural arrangement of Cx43 in gap junction membranes.

The crude serum collected from each immunized rabbit was found to be 5- to 15-fold more reactive towards immobilized peptides than the corresponding preimmune serum (Table 1). Three of the site-directed antibodies (CL-100, EL-186 and CT-360) were found to be highly reactive towards the Cx43 protein on Western blots, at serum dilutions of 5000-, 2000- and 20000-fold, respectively. However, the reactivity of EL-46 and CT-237 antibodies to Cx43 on Western blots was not as high. Although there

Table 1. *Characterization of site-directed antibodies*

Antibody	Peptide sequence	Reactivity to peptide (cts min^{-1} immune serum / cts min^{-1} preimmune)	Relative reactivity to peptide	Relative reactivity to Cx43 in immunoblots
EL-46	46–76	14.8	++++	++
CL-100	100–122	9.8	+++	++++
EL-186	186–206	5.1	++	++++
CT-237	237–259	11.1	+++	++
CT-360	360–382	10.6	+++	++++

The Cx43 peptides synthesized were based on the protein sequence obtained from Beyer *et al.* (1987). The prefixes used for the site-directed antibodies indicate the location of the epitopes with respect to the lipid bilayer: EL, extracellular loop; CL, cytoplasmic loop; and CT, carboxy terminus. The number associated with each antibody denotes the amino acid closest to the amino terminus. Reactivity against the peptides was measured as binding of the immune serum to immobilized synthetic peptides by a radioimmune assay In all cases the respective preimmune serum was assayed as a measure of non-specific binding The relative ability of the antibodies to bind to the Cx43 protein was determined by SDS–PAGE in conjunction with immunoblotting.

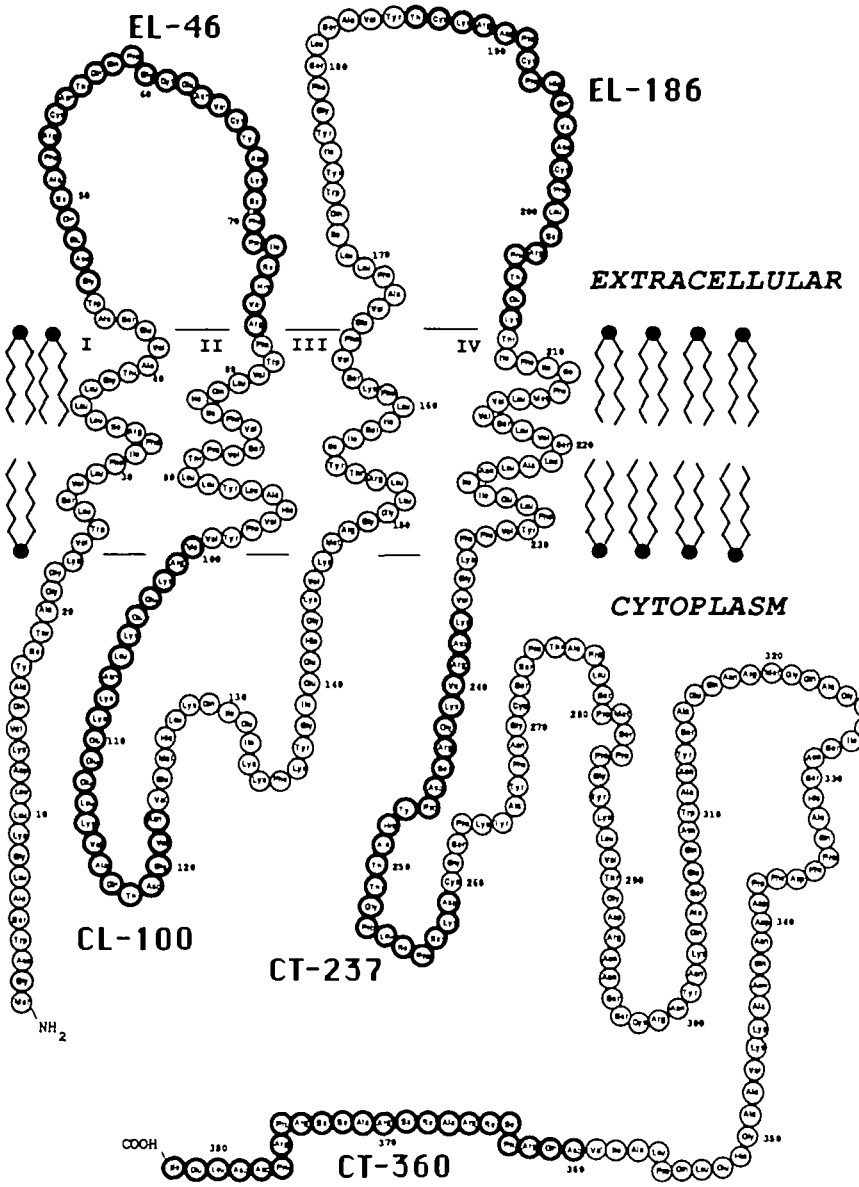


Fig. 1. A model of Cx43 depicting the locations of the peptide segments synthesized (in bold) and the names of the corresponding antibodies raised. This model illustrating how the polypeptide threads through the membrane four times, yielding two extracellular loops and a cytoplasmic loop was constructed based on proposed models for Cx32 (Paul, 1986; Zimmer *et al.* 1987; Milks *et al.* 1988; Goodenough *et al.* 1988) and recent models of Cx43 (Beyer *et al.* 1987; Yancey *et al.* 1989). The Cx43 peptides that we chose to raise antibodies against represented two highly conserved domains predicted to be exposed to the extracellular surface and three segments postulated to be accessible to the cytoplasm (Beyer *et al.* 1987; Yancey *et al.* 1989). The transmembrane helical segments are denoted by roman numerals.

was considerable sequence homology between some of the Cx43 peptides (46–76 and 186–206) and segments of the Cx32 polypeptide chain, none of the antibodies raised cross-reacted with Cx32 on Western blots (unpublished data).

Site-directed antibodies were purified against virtually homogeneous (as defined by HPLC) synthetic peptides by passing immune serum through peptide-coated MAC 25 membrane filters. One antibody (CT-237) could not be purified using this technique, suggesting that the immobilized peptide did not present the proper conformation for antibody binding.

Topology of Cx43

Proteolysis of cardiac gap junctions. Isolated cardiac gap junctions were subjected to proteolysis and the resulting fragments were analyzed by immunoblotting. As reported previously (Manjunath *et al.* 1984*b*; Yancey *et al.* 1989), SDS–PAGE of heart gap junction membrane preparations yielded a broad band at $43\times10^3 M_r$ as well as three breakdown products of 35, 33 and $31\times10^3 M_r$ (Fig. 2, CB lane a), which are believed to have lost carboxy-terminal fragments (as shown below). The additional bands above $43\times10^3 M_r$ represent either aggregates of Cx43 or impurities in the gap junction membrane preparation. Immunoblotting with affinity-purified CL-100, EL-46 and EL-186 antibodies resulted in the labeling of the major band at $43\times10^3 M_r$ and also the breakdown products at 35, 33 and $31\times10^3 M_r$ (Fig. 2, CL, EL lane a). In blots treated with purified CT-360 antibody (specific for the C-terminal tip) the $43\times10^3 M_r$ band, but not the breakdown products, were labeled, indicating the loss of carboxy-terminal domains in the bands seen at 35, 33 and $31\times10^3 M_r$ (Fig. 2, CT lane a).

When isolated junctions were completely digested with endoproteinase Lys-C (which cleaves proteins at the carboxy side of lysine residues) a major membrane-bound fragment was resolved at $15\times10^3 M_r$ and a second more diffuse band at approximately $21\times10^3 M_r$ (Fig. 2, CB lane b).

The EL-46 site-directed antibody labeled a band at $15\times10^3 M_r$ as well as some incompletely endoproteinase Lys-C-digested or aggregated fragments at 28, 30 and $38\times10^3 M_r$, but no bands around $21–22\times10^3 M_r$ (Fig. 2,

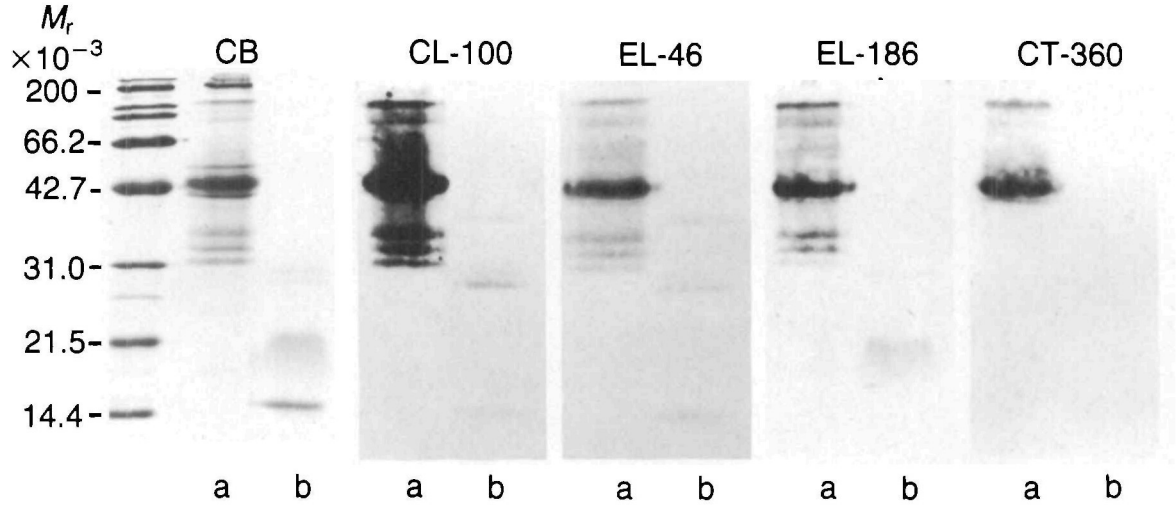


Fig. 2. Analysis of polypeptides from untreated and endoproteinase Lys-C-digested gap junction membranes that bind site-directed antibodies. Undigested gap junction membranes (lane a) or endoproteinase Lys-C-digested gap junction membranes (lane b) were washed and electrophoresed on a 12% SDS–polyacrylamide gel. The polypeptides were either stained with Coomassie Blue (CB) or transferred to nitrocellulose paper and treated with the affinity-purified site-directed antibodies indicated. The molecular weight standards (described in Materials and methods) are shown in the far left lane of the Coomassie Blue-stained gel.

lane b). A similar, but weaker, pattern of labeling was observed for the CL-100 antibody (Fig. 2, lane b). However, the EL-186 antibody only labeled the broad 21×10^3 M_r fragment (Fig. 2, lane b). Endoproteinase Lys-C digestions of isolated junctions resulted in the loss of CT-360 antibody binding. These results suggest that the binding site for the EL-46 and EL-186 antibodies are protected from endoproteinase Lys-C digestion but epitopes exposed on the cytoplasmic side are more easily accessed. The epitopes for EL-46 and EL-186 antibodies were also retained when junctional membranes were digested with trypsin (unpublished data).

Immunogold labeling of isolated junctions. Immunogold-labeling studies were performed in order to explore further the accessibility of the various domains of the Cx43 protein in gap junctions. Both the CT-360 and CL-100 antibodies could be used to immunogold label the cytoplasmic surface of isolated junctions (Fig. 3A,B). When preimmune sera were used only a few scattered gold particles were observed along the membrane surface (Fig. 3C). The EL-186 antibody was shown to be virtually ineffective in immunogold labeling of gap junction membrane preparations (Fig. 3D). In several instances, however, gold particles were found at the tips of the gap-junctional plaques (unpublished data). These results confirm that only the two Cx43 epitopes (amino acids 100–122 and 360–382), predicted to be exposed along the cytoplasmic surface, are accessible for antibody binding in structurally intact gap junctions. In such preparations, the protease-protected second extracellular loop (amino acids 186–206) is inaccessible, except possibly at the broken ends of the plaques.

Labeling of extracellular loops only under specific conditions. Neonatal cardiac myocytes can effectively be isolated and grown in culture (Mark and Strasser, 1966; Burt, 1982; Yancey *et al.* 1989) and can couple through gap junctions to adjacent cells (Burt and Spray, 1988; Yancey *et al.* 1989). Thus, primary cultures of neonatal cardiac myocytes were employed in an attempt to label Cx43 in gap junctions using antibodies directed towards the extracellular loops. Conventional methods of permeabilizing cell pairs with ethanol prior to labeling with the EL-46 antibody were unsuccessful (Fig. 4A). When myocytes were fixed with paraformaldehyde (but not permeabilized) and labeled with the EL-46 antibody, small fluorescent spots were occasionally found dispersed on the cell surface, but no labeling could be found at the cell boundaries (Fig. 4B). Similar results were found when ethanol-treated or paraformaldehyde-fixed cells were stained with the EL-186 antibody (unpublished data). However, after treatment with urea at high pH, the EL-46 (Fig. 4C) and EL-186 (Fig. 4D) antibodies both label junctional plaques at locations of myocyte cell–cell contact. It appears that in the alkali/urea-treated myocyte pairs the apposed membranes in gap junctions were split, allowing the EL-46 and EL-186 antibodies to access the extracellular domains. The fact that the CT-360 antibody also labels punctate structures at points of cell–cell apposition in alkali/urea-treated myocytes (unpublished data) supports the conclusion that the EL-46 and EL-186 antibodies are reacting specifically with Cx43-containing gap junction plaques.

Only myocytes expressed the Cx43 protein

Immunofluorescent labeling studies were performed to test for the distribution of Cx43 protein in cardiac myocyte cultures. Labeling of three-day-old cultured cardiac cells with CT-360 antibody clearly demonstrated the presence of punctate zones of fluorescence at locations of myocyte cell–cell contacts (Fig. 5C). In double-labeling experiments the same cells were labeled with an anti-myosin monoclonal antibody that stained the myofibrils of the cardiac myocytes but did not label the fibroblasts present in the culture (Fig. 5B). It is clear that Cx43-containing junctions are formed between adjacent myocytes but not generally between myocytes and fibroblasts. However, in a few cases, we have obtained Cx43 staining at sites of myocyte–fibroblast apposition (unpublished data). Fibroblasts that were in contact with adjacent fibroblasts did not stain with the CT-360 antibody.

The same structures are labeled by different anti-Cx43 antibodies

In other double-labeling studies, gap junctions were first labeled with an antibody (CL-100) against the cytoplasmic loop and then with a second antibody (CT-360) against the carboxy terminus (Fig. 6). The immunofluorescent labels

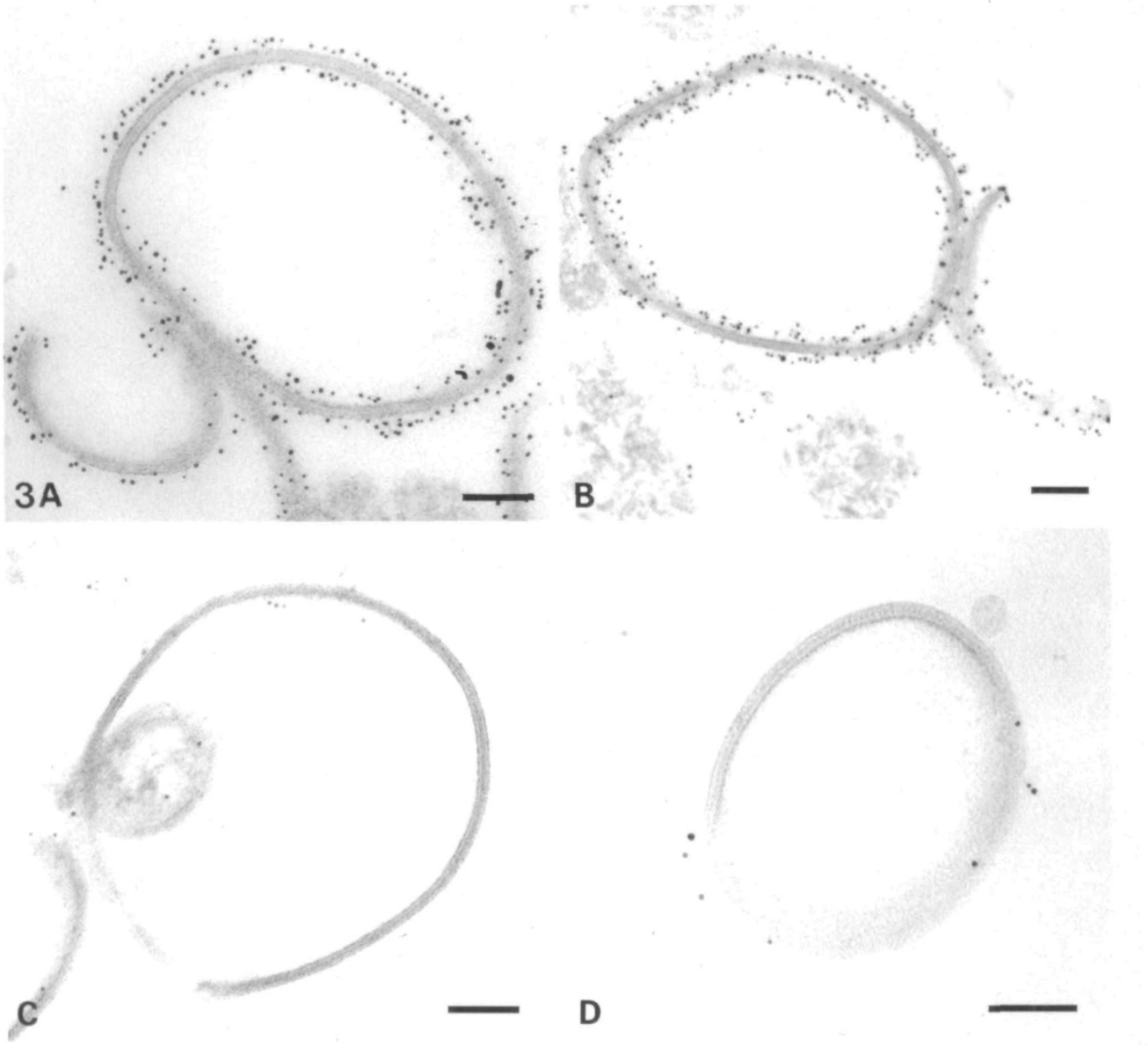


Fig. 3. Transmission electron micrographs of isolated gap junction membranes immunogold labeled after exposure to anti-Cx43 site-directed antibodies. Immobilized gap junction membranes were treated with CT-360 (A) or CL-100 (B) antibodies followed by goat anti-rabbit IgG conjugated to 5 nm gold particles. Both of these antibodies intensely labeled the cytoplasmic surface of the isolated gap junction plaques. Little labeling was found when a preimmune serum was used as the primary antibody (C). When EL-186 antibody was used only a few gold particles were found associated with junctional plaques (D). Bar, 0.1 μm.

were found to colocalize, irrespective of whether the cells were permeabilized with ethanol or paraformaldehyde/Triton X-100 (Fig. 6).

Both phosphorylated and dephosphorylated forms of Cx43 are recognized by CT-360 antibody

Since the CT-360 antibody was raised against a serine-rich, nonphosphorylated, synthetic peptide, the possibility existed that the CT-360 antibody only recognized the nonphosphorylated form of Cx43. In order to test this possibility, isolated cardiac gap junctions were digested with alkaline phosphatase before probing with the CT-360 antibody (Fig. 7). In untreated gap junctions a broad Coomassie Blue-Staining band was found at $43\times10^3\,M_r$ (Fig. 7, CB lane a); however, after alkaline phosphatase treatment of the junctions the major band decreased in size to $40\times10^3\,M_r$ (Fig. 7, CB lane b). The faint but sharp band at $43\times10^3\,M_r$ that remained after alkaline phosphatase treatment (Fig. 7, CB lane b) was determined by immunoblotting to be actin (unpublished data). Alkaline phosphatase digestion had no effect on the migration of the three major breakdown fragments of Cx43 at 35, 33 and $31\times10^3\,M_r$ (Fig. 7, CB, compare lanes a and b). Immunolabeling with the CT-360 antibody resulted in a broad spectrum of bands from $40–45(\times10^3)\,M_r$ to stain in the undigested preparation of gap junctions (Fig. 7, CT lane a). However, the CT-360 antibody bound principally to the major band at $40\times10^3\,M_r$ in the alkaline phosphatase-treated sample and failed to bind to the sharp band at $43\times10^3\,M_r$ (Fig. 7, CT lane b). Faint CT-360 antibody binding to the presumed dimer of Cx43 was observed in both the untreated and alkaline phosphatase-treated membranes. The $40\times10^3\,M_r$ band produced by alkaline

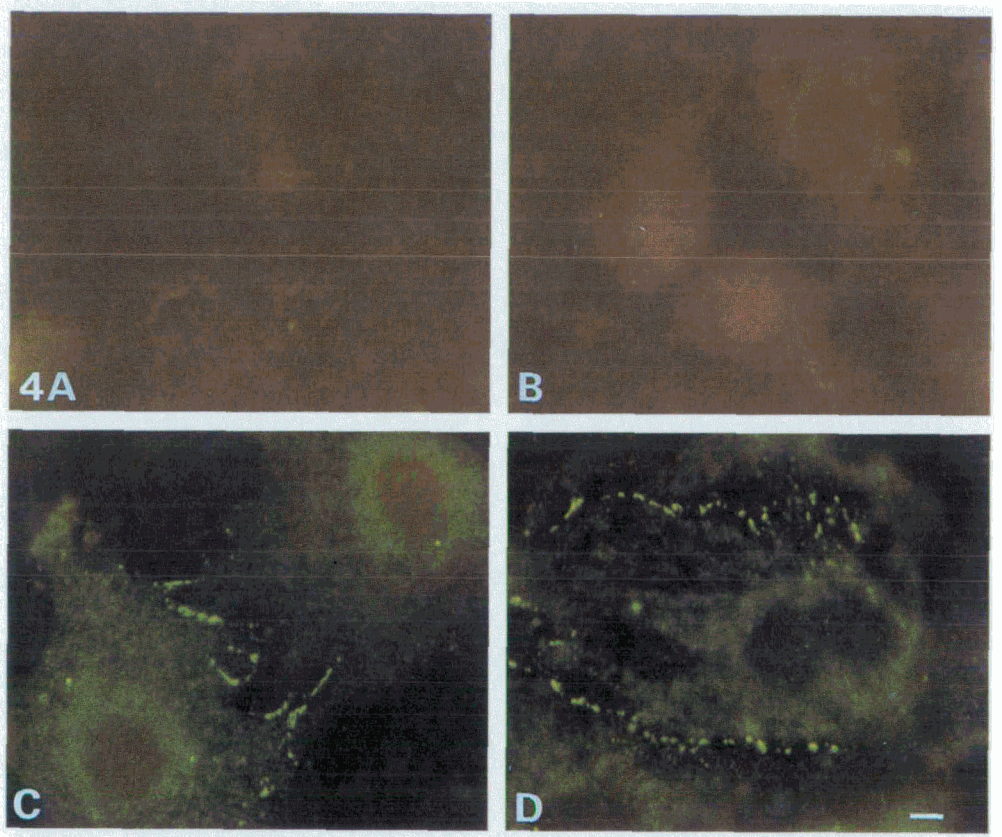
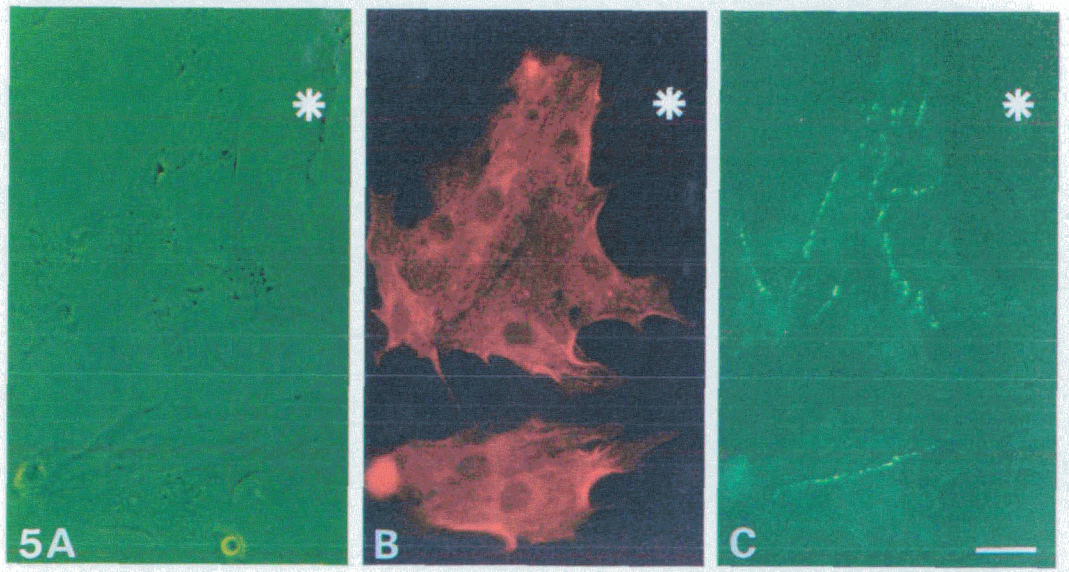
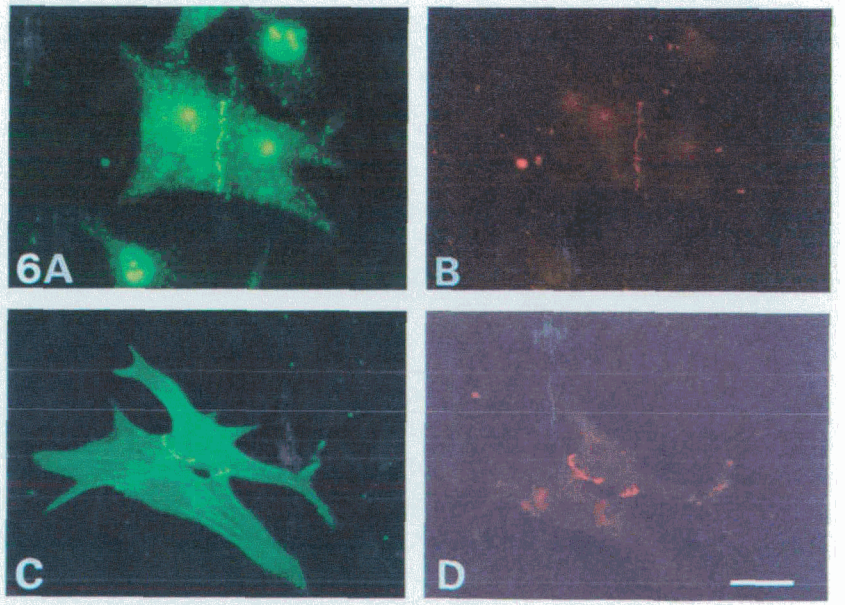


Fig. 4. The accessibility of Cx43 extracellular loops to immunolabeling in cultured neonatal cardiac myocytes. Cultured myocytes were permeabilized with ethanol (A), fixed with paraformaldehyde (B) or fixed, washed and alkali/urea treated (C) prior to exposure to EL-46 anti-Cx43 antibody. After washing, bound antibody was detected using an anti-rabbit IgG antibody conjugated to fluorescein. In D, fixed, alkali/urea-treated cells were stained using EL-186 as the primary antibody. Immunoreactivity was localized to locations of cell–cell apposition only when fixed cells were treated with alkali/urea. Bar, 10 μm.

Fig. 5. Localization of myosin and Cx43 protein in neonatal cardiac myocytes. Permeabilized, cultured neonatal myocytes (A) were treated with an anti-myosin monoclonal antibody followed by a goat anti-mouse IgG antibody conjugated to rhodamine (B). Alternatively, these same myocyte cells were treated with a CT-360 anti-Cx43 antibody. After washing, bound antibody was detected by a goat anti-rabbit IgG fluorescein conjugate (C). The asterisk denotes a fibroblast cell, not labeled with either the anti-myosin or anti-Cx43 antibody. Anti-Cx43 immunoreactivity was found primarily between apposed myocytes. Bar, 50 μm.

Fig. 6. Colocalization of anti-Cx43 site-directed antibodies to the same junctional plaques in neonatal cardiac myocytes. Cultured myocytes were either fixed in paraformaldehyde and permeabilized with Triton X-100 (A,B) or directly permeabilized with ethanol (C,D). Cells were labeled indirectly with CL-100 antibody followed by a goat anti-rabbit IgG–fluorescein conjugate (A,C), washed and labeled with CT-360 antibody directly conjugated to rhodamine (B,D). The cells were prepared for fluorescent microscopy. Myocytes stained with the CL-100 antibody exhibited varying types of background fluorescence depending on the labeling procedure used. Bar, 50 μm.





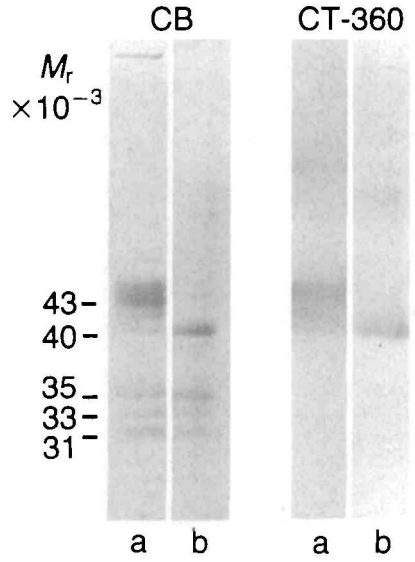


Fig. 7. The effect of alkaline phosphatase digestion of gap junctions on CT-360 antibody binding. Undigested gap junction membranes (lane a) or alkaline phosphatase-digested gap junction membranes (lane b) were electrophoresed on a 10% SDS–polyacrylamide gel containing 0.4% bisacrylamide and either stained with Coomassie Blue (CB) or transferred to nitrocellulose paper. The Western blot was labeled with the CT-360 antibody.

phosphatase digestion was not the result of proteolysis at the amino terminus of Cx43, as this domain was found to be immunologically intact (unpublished data). These results clearly indicated that the CT-360 antibody recognized many phosphorylated and dephosphorylated forms of the Cx43 protein.

Discussion

Topology of Cx43

We have extended the understanding of the structure and organization of the Cx43 gap junction protein by using several new site-directed antibodies. We have found that, in agreement with expectations based on the topology of the Cx43 protein in gap junction membranes deduced from previous work, antibody binding to the two potential extracellular loops was not eliminated after digestion with several proteolytic enzymes (trypsin, endoproteinase Lys-C and endogenous proteases). This is believed to be due to the inability of proteases to penetrate the narrow extracellular gap between apposed membranes in gap junctions. Support for the structural intactness of gap junctions under these conditions was provided by studies of Manjunath *et al.* (1985) in which gap junctions that were subjected to proteolysis by endogenous proteases maintained their double-membrane appearance. We show here that when isolated gap junctions are digested with endoproteinase Lys-C, the EL-46 antibody bound to a 15×10^3 M_r membrane-protected fragment while the EL-186 antibody labeled a slightly larger fragment of 21×10^3 M_r. This is consistent with the EL-46 antibody recognizing an epitope within the domain that encompasses the first and second proposed transmembrane helices and the EL-186 antibody binding to an epitope within the membrane-protected segment containing the third and fourth helices. The failure to label intact junctional membranes with the EL-186 antibody is again consistent with the 186–206 amino acid segment being masked within the extracellular gap. In several cases the tips of the junctional plaques were labeled, suggesting that, as might have been predicted, extracellular domains could be exposed in such areas.

Immunogold-labeling studies using the CL-100 and CT-360 antibodies have demonstrated that the 100–122 and 360–382 amino acid segments are available on the cytoplasmic surface of the membrane for antibody binding. In addition, these two antibodies can be colocalized to the same junctional plaques. The epitope for CL-100 antibody binding was lost after trypsin treatment (unpublished data) but was partially retained after endoproteinase Lys-C digestion. Therefore, one or more tryptic sites must be located within or near the 100–122 segment and a major site of endoproteinase Lys-C digestion probably lies beyond the CL-100 antibody binding site. All proteolytic digestions of Cx43 removed the CT-360 antibody binding site, confirming the susceptibility of the carboxy terminus to proteases, as proposed by other investigators (Manjunath *et al.* 1984*b*; Manjunath *et al.* 1985; Yancey *et al.* 1989). These results clearly support the idea that the Cx43 polypeptide threads through the membrane four times yielding accessible proteolytic sites within the cytoplasmic loop domain and along the carboxy terminus. The reactivity of the anti-peptide antibodies also puts clear constraints on the portions of the polypeptide chain that are protected by the membrane itself.

Cx43 is phosphorylated

It is interesting to note that all the Cx43 site-directed antibodies label a broad spectrum of bands (within the range of $40–45\times10^3$ M_r) on Western blots of gap junction membranes. In previous reports (Manjunath *et al.* 1984*b*; Yancey *et al.* 1989), speculations were made that the multiplicity of bands reflected proteolysis at the very carboxy-terminal tip of the polypeptide. However, we show here that the CT-360 antibody, raised against the carboxy-terminal tip (360–382) of Cx43, binds to this wide spectrum of bands, thus excluding the possibility of significant proteolysis within this carboxy-terminal domain. It is possible that the CT-360 antibody and other site-directed antibodies, cross-react with a protein highly homologous to Cx43. At present there is no direct evidence for the existence of such a protein. An alternate view is that the Cx43 protein in gap-junctional plaques has undergone varying degrees of post-translational modifications (i.e. phosphorylation).

We present evidence that indicates that the CT-360 antibody does in fact recognize a variety of phosphorylated forms of the Cx43 protein within the $40–45\times10^3$ M_r range. Moreover, there was no migrational change in the major breakdown fragments at 35, 33 and 31×10^3 M_r when digested with alkaline phosphatase, suggesting that the carboxy-terminal 8×10^3 M_r fragment contains most, or all, of the sites of phosphorylation. These breakdown products have lost the epitope for binding the CT-360 antibody but not the segments required for EL-46, CL-100 and EL-186 binding. Whether the carboxy tip is the only site that is phosphorylated is uncertain. It is possible that the large breakdown fragments are phosphorylated at sites that are inaccessible to the alkaline phosphatase. In addition, it is not clear how many phosphate residues need to be removed to make a cleavage resolvable by SDS–PAGE. On the basis of the primary sequence of Cx43, the 360–382 segment alone contains six serine residues (Beyer *et al.*

1987). Four of these serine residues are positionally identical to carboxy-terminal serine residues in a lens fiber Cx46 protein (Beyer *et al.* 1988). Exact conservation of these residues is consistent with the idea that these serine residues may be important in regulating junctional activity. Interestingly, within the last 20 amino acid residues of the Cx43 sequence there are three consensus sites for possible calmodulin-dependent phosphorylation (Miller *et al.* 1988). These potential phosphorylation sites are not found in the liver Cx26 and Cx32 gap junction proteins. However, previous studies have shown that in the liver the catalytic subunit of cyclic AMP-dependent protein kinase is directly involved in the phosphorylation of a serine residue on Cx32 gap junction protein and in the modulation of channel conductance (Spray *et al.* 1985; Saez *et al.* 1986), but the mechanism or role of phosphorylation in Cx43 is not known at present.

Heterologous junctions

Gap-junctional plaques were clearly labeled with CL-100 and CT-360 antibodies at areas of cell–cell contact in neonatal myocyte cell cultures. Sometimes, but not always, immunofluorescently labeled plaques were observed at locations of myocyte–fibroblast apposition (unpublished data). This is reminiscent of recent work by Rook *et al.* (1989), who have identified not only electrically coupled myocytes but also myocytes that were coupled to fibroblasts. These heterologous gap junction channels were believed to be composed of two different connexins that possess homologous extracellular domains. Our results suggest that in some cases myocytes may contribute Cx43 in the form of hemichannels for gap junction formation with fibroblasts.

Is all Cx43 in plaques?

In neonatal cardiac myocyte cultures the presence of Cx43 on the cell surface was occasionally detected by the EL-186 and EL-46 antibodies in immunofluorescent labeling studies. It is possible that such labeling detects partially assembled gap junction membrane structures that are remnants from the cardiac tissue prior to culturing. It has also been claimed that gap junction degradation and disappearance in cultured adult cardiac myocytes is a slow process (Severs *et al.* 1989). Alternatively, Cx43 protein located on the cell surface of neonatal myocytes may represent partially assembled junctional plaques that are in the process of being sorted as hemichannels to locations of cell–cell contact for the complete channel formation. Assembled gap junctions do not react with the EL-46 or EL-186 antibodies unless the myocyte junctions are disrupted (split) with an alkali/urea treatment. Alkali/urea treatments of gap junctions have been reported to break hydrogen and hydrophilic bonds between connexons, resulting in the formation of single membrane structures (Manjunath *et al.* 1984*a*,*b*; Zimmer *et al.* 1987; Goodenough *et al.* 1988).

In summary, five site-directed antibodies have been raised against strategically chosen domains of the Cx43 protein. These antibodies have been used to confirm the proposed model of Cx43 where the polypeptide chain threads through the membrane four times, yielding two extracellular loops and a protease-sensitive cytoplasmic loop. These antibodies should prove to be useful immunological probes in studying the assembly and disassembly of gap junctions in cardiac tissue. In addition, as the emphasis of Cx43 studies moves towards a more analytical dissection of protein function, these antibodies may prove to be extremely valuable tools for studying native and mutated Cx43 protein in expression systems.

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